

Endocrinological Manipulations and Initial Maternal Behaviour
in the Virgin Rat (*Rattus norvegicus*)

by

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Abstract

Steroidal hormones are known to mediate maternal behaviour by influencing specific neural structures that are responsible for initial onset and maintenance of this behaviour. Contrary to typical extirpation and replacement strategies, this study examined hormonally intact animals to evaluate the specific effects of hormonal enhancement in conjunction with endogenous fluctuations on maternal behaviour. Rats were given a regimen of estrogen and progesterone hormones via osmotic pumps and subcutaneous injections to mimic natural pregnancies. Maternal behaviours were recorded for a twenty-four hour observation period either immediately after parturition or after a sensitization period to foster pups. Neural tissue was removed from the medial preoptic area after the observation period and prepared for future analysis with electron microscopy. Behavioural results from this study revealed that initial behaviours are not influenced in exogenously enhanced virgins compared to virgin and pregnant controls. These results suggest the significance of neural moderation on the onset and magnitude of maternal behaviour.

Keywords

ELISA

Endocrine

Estrogen

Hormones

Learning

Limbic System

Maternal Behaviour

Maternal Enhancement

Medial Preoptic Area

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Introduction

1 General Introduction

The aim of this thesis was to investigate the degree to which certain hormones elicit and influence maternal behaviours in rats. The following sections review the relevant literature and seminal studies that examined maternal behaviour, endocrinology, and neural circuitry. This past research forms the basis and rationale for the current project's design and hypotheses.

2 Maternal Behaviour

Maternal behaviour encompasses species-specific responses to offspring, which ensures the health and protection of young and the propagation of the species (Rosenblatt and Snowden, 1996). In mammalian species, the expression of maternal behaviour is a complex and significant learning event (Kinsley and Amory-Meyer, 2011; Li and Fleming, 2003). Initiation may occur without previous experience, but retention is often critically based on the stimuli and biofeedback where interference may lead to inhibition of behaviours and the loss of the offspring (Li and Fleming, 2003; Orpen and Fleming, 1987). Investigations of maternal behaviour in non-human mammals provide insight into the complex nature of the relationship between mother and child (Kim et. al, 2010; Kinsley and Meyer, 2010). Previous research has investigated the modulation, direction, and integration of internal and external stimuli necessary for the development of maternal behaviour. The onset and maintenance of this behaviour is reliant on an intricate balance of steroidal, neural, and external cues (Fleming and Corter, 2002; Numan, 2012; Rosenblatt, 1967).

In the absence of the appropriate cues and stimuli, the expression of maternal behaviour is inhibited partially or completely. Specifically, in the albino Wistar rat (*Rattus norvegicus*), near-complete inhibition of maternal responsiveness is observed until late-pregnancy (Rosenblatt and Lehrman, 1963; Wiesner and Sheard, 1933). Prior to parturition, commonly referred to as birth, the rat is neophobic and will be averse to young pups within her vicinity. Behaviours exhibited by pup-averse rats include avoidance, pup-burying, and pup-cannibalization (Rosenblatt, 1967; Mann and Bridges, 2001). Using graduated exposure, a neophobic virgin (nulliparous) rat can be sensitized to foster pups (Rosenblatt, 1967). After a period of five to seven days, nulliparous animals will begin to illicit maternal behaviours such as grooming, hovering, and retrieval. In order to prevent pup cannibalization and to provide adequate stimuli, nulliparous animals in the current study were sensitized to pups for the minimal period (five days) before free-exposure.

Normally, a pregnant rat begins to express maternal behaviour near the end of a twenty-one day gestational period (Rosenblatt and Lehrman, 1963). As early as day eighteen, the previously pup-averse rat will now accept foster pups presented to her, prepare a nest with available material, and will begin to exhibit aggressive behaviours towards intruders (Mayer and Rosenblatt, 1984). Once parturition begins, the rat, now called a dam, will deliver her pups, ingest their placentas, clean pups through licking behaviour, and place them into her nest until her full litter is born (Fleming, O'Day, and Kraemer, 1999). Within a day of parturition, the dam will display a full range of maternal behaviours, which include nursing, crouching, anogenital licking, and pup retrieval. The onset of these behaviours will occur immediately upon parturition and, unlike most mammals, require no previous experience in birth or maternal care (Fleming and Rosenblatt, 1974). These behaviours do, however, require the presence of normally circulating hormones, active brain regions, and appropriate pup stimuli.

3.1 Prolactin

Extensive research has been conducted on the significance of hormones produced by the pituitary gland, the hypothalamic nuclei, the ovaries and their respective roles in maternal responsiveness. The pituitary gland produces the lactogenic hormone prolactin, which increases and decreases rhythmically during diurnal and nocturnal cycles until mid-pregnancy (Mann and Bridges, 2001; Bridges, 2015). During the latter half of a rat's pregnancy, prolactin remains at low concentrations, while placental lactogens (similar to prolactin) begin peaking. Research has established that this complex cycling has a strong stimulatory effect on maternal responsiveness, especially in primiparous dams (Anderson, Gratten, van der Ancker, and Bridges, 2006). Early researchers failed to establish the finding that implied prolactin involvement in maternal behaviour; however, further investigations determined the importance of steroidal priming in combination with prolactin injections. Steroidal priming requires the exogenous exposure of estrogens and progesterone in order to catalyze the influence of prolactin in ovariectomized animals (Mann and Bridges, 2001). The introduction of exogenous prolactin along with steroidal priming has been shown to reduce latency to onset of maternal behaviour significantly from six days to two days in nulliparous rats (Krasnegor and Bridges, 1990).

In 1990, Bridges and Ronsheim utilized a dopamine agonist, brocriptine, to inhibit endogenous release of prolactin from the pituitary. Exogenous replacement of prolactin induced maternal behaviours only in the presence of steroidal priming as previous research has indicated. The study confirmed the necessity of endogenous prolactin levels in nulliparous animals and led to a subsequent series of experiments that examined the site of action and modulation of prolactin on

maternal behaviour (Bridges, Numan, Ronsheim, Mann, and Lupini 1990). In nulliparous rats, Bridges investigated varying dosages of exogenous prolactin through intracerebroventricular infusions, directly into the medial preoptic area (MPOA), and subcutaneous injections. Each investigation included the presence or absence of exogenous steroidal priming and endogenous prolactin was suppressed in each subject using brocriptine. Subcutaneous injections failed to exhibit an effect on the onset of maternal behaviour at dosages that were sufficient in ventricular infusions. Significantly small dosages of prolactin, insufficient to induce maternal behaviours through ventricular infusions were, however, sufficient when directly infused into the MPOA and clearly induced onset to maternal behaviour. These experiments, once again, confirmed the necessity of steroidal priming and highlighted a clear sensitivity of prolactin activity within the central nervous system.

A study conducted by Bridges and Dunkell (1987) utilized prolactin in conjunction with progesterone priming in the absence of exogenous or endogenous levels of estrogens. The study showed maternal behaviours in the absence of estrogen but highlighted the requirement for the other ovarian hormone progesterone. This result identified a potentially complex relationship between ovarian hormones and prolactin during the onset of maternal behaviour.

3.2 Oxytocin

Similar to prolactin, the hypothalamic hormone oxytocin, which is stored and released in the posterior pituitary, requires steroidal priming to induce maternal responsiveness to foster pups (Pederson and Prange, 1979). Oxytocin has been demonstrated to be a significant mediating hormone in conjunction with estrogen, but only through direct injection into the cerebral ventricles or the MPOA (D'Cuna, King, Fleming, and Levy, 2011; Pederson and Prange, 1979;

Pederson, Caldwell, Walker, Ayers, and Mason, 1994). Pharmacological blockage of oxytocin will inhibit maternal behaviours and genetic knockouts have been shown to result in high pup mortality due to poor or no milk production. This suggests a pertinent role of oxytocin in not only mediating behaviours, but in other physiological processes as well (Champagne, Diorio, Sharma, and Meaney, 2001; Macbeth, Stepp, Lee, Young, and Caldwell, 2010). These studies suggest oxytocin plays a more peripheral role in the onset of maternal behaviour, but provides further evidence for the importance of estrogen.

3.3 Estrogen and Progesterone

Arguably, the two most important hormones that mediate maternal behaviour are the ovarian estrogens and progesterone. A rat's pregnancy is maintained by these steroidal hormones for the duration of gestation until parturition (Mann and Bridges, 2001). In 1984, Bridges conducted a study to investigate the effects of dosage and duration of steroidal hormones together and separately on maternal behaviour. The study included the quantification and comparison of endogenous and exogenous levels of steroidal hormones. Since estrogen concentrations in a rat's blood are only measurable in picograms per millilitre, this analysis required the use of a radioimmunoassay (RIA). Progesterone concentrations which are higher, were measureable with an enzyme-linked immunosorbent assay (ELISA) in nanograms millilitre. According to the results of the assays, endogenous concentrations of estrogens were shown to remain low until parturition approached (day eighteen onwards), and then increase dramatically. Concentrations of progesterone, however, remained high during pregnancy and declined to immeasurable levels just before parturition. Bridges examined the dosage and effect of exogenous hormonal levels using subcutaneous implants to permit slow release of the hormones, which mimicked a more natural circulation. Utilizing this approach, Bridges was able to approximate endogenous levels

of estrogen and progesterone and induce rapid onset to express maternal behaviour in nulliparous rats. The duration of progesterone priming reduced latency to maternal behaviour from four days to one day in animals which were primed for thirteen days rather than five. In the absence of progesterone withdrawal, either through continuous injections or by maintenance of implants, maternal behaviours were inhibited (Seigal and Rosenblatt, 1975).

In combination with estradiol benzoate (estradiol-17 β), a form of estrogen, progesterone will rapidly shorten the onset to maternal behaviours in pregnancy-terminated and nulliparous rats (Doerr, Siegal, and Rosenblatt, 1981). Supplementation of estradiol-17 β alone is insufficient to induce rapid maternal responsiveness compared to controls, indicating estrogen alone may not be responsible for onset of maternal behaviour (Bridges, 1984). The efficacy of the combination of estradiol-17 β and progesterone is sensitive to the timing and duration of supplementation suggesting the decline of progesterone primes the rat's sensitivity to estrogens (Bridges and Russell, 1981; Bridges, 1984).

Similar to prolactin and oxytocin, direct infusion of estrogen into the MPOA stimulates maternal behaviours (Numan, Rosenblatt, and Komisaruk, 1977). Direct infusion of progesterone, however, does not. Further, direct infusion of progesterone after pregnancy termination also fails to inhibit maternal behaviour. This contrasts with systemic injections of progesterone, which will induce behaviours, indicating a different site of action for this hormone (Numan, 1980).

The complex and sensitive nature of the interaction between neural circuitry and ovarian hormones are evident but not completely understood. The roles of each hormone have been individually examined and are deemed important, and usually necessary for the rapid onset of maternal behaviours such as retrieval, nursing, and grooming. Research has shown, however,

that hormone changes are not required for the onset of maternal behaviour, or for their maintenance (Rosenblatt, 1967). According to more recent literature, maternal pathways and structures within the brain are significant mediators in this role (Fleming and Corter, 2002). Due to their significance, the next section details the pertinent mediating structures, neural circuitry, and their respective roles in maternal behaviour.

4 Maternal Neural Circuitry

Evidence purporting a non-hormonal basis for the onset and maintenance of maternal behaviours was provided by Rosenblatt in 1967. In this important experiment, he examined the response of groups of intact, ovariectomized, hypophysectomized, female nulliparous rats along with intact and castrated males to foster pups after a sensitization period. After continuous exposure of five to seven days, most animals, regardless of group, displayed complete maternal behaviours at different latencies. These results indicate an influence of the presence of hormones on the onset of maternal behaviours but that they are not necessary.

4.1 Medial Preoptic Area

Fundamental research into the neural basis of maternal behaviour began in the 1970s. Early studies utilized surgical lesioning techniques to determine pertinent regions and pathways within the brain. The MPOA and other components of the limbic system were found to be critical to the onset of maternal behaviour (Numan, 1974; Smother, Hennessy, and Levine, 1977; Terkel, Bridges, and Sawyer, 1978). The MPOA contains a high density of estrogen receptors and is located in the anterior hypothalamus, above the pituitary (Frick, Kim, Tuscher, and Fortress, 2015; Rainbow, Parsons, MacLusky, and McEwen, 1982; Rosenblatt and Ceus, 1988). Direct chemical lesioning of the MPOA and surrounding structures, such as the substantia innominata

and pathways to the ventral tegmental area (VTA) severely diminished established maternal behaviours in lactating dams (Kuroda and Numan, 2014; Numan, Corodimas, Numan, Factor, and Piers, 1988). Chemical lesions to the MPOA were followed up by histochemical tracing, providing evidence that the cell bodies within the MPOA were damaged, while leaving the axons and tracts intact (Numan et al., 1988). Disrupted maternal behaviours through usage of radiofrequency or electrolytic lesioning of the MPOA were specific to nest building, retrieval, and nursing behaviours (Numan, 1988). An operant conditioning experiment that involved bar-pressing for pups, reinforced the deficits of maternal behaviour displayed by electrical lesioning of the MPOA, and also indicated the lateral amygdala (LA) as an important mediating structure (Lee, Clancy, and Fleming, 2000). Electrical stimulation of the MPOA of virgin rats resulted in a preference for pup-associated environments (Morgan, Watchus, Milgram, and Fleming, 1999). The electrical stimulation, along with previous evidence of hormonal stimulation of the MPOA presented in the endocrine section of this study, provides further indication of the MPOA's involvement in maternal responsiveness.

Further lesion based studies examined the MPOA's efferent connections and relevance to maternal behaviours. Lesions and tract-tracing techniques have examined projections from the MPOA to the lateral preoptic area (LPOA) and the VTA. These results provided visual evidence for lateral MPOA estrogen efferents in this pathway (Numan, 1988). Estrogen and progesterone activate or inhibit the expression of specific genes in the MPOA, which can have immediate or delayed cellular effects (Beato and Klug, 2000).

The profound influence of the MPOA on maternal behaviour, along with the direct effects of steroidal hormones within this region, is the rationale for the removal of MPOA tissue as detailed in the appendices for future analysis. In combination with behavioural data, the structural

analysis of the MPOA will provide a more comprehensive representation of the interactions implicated by the literature.

4.2 Olfactory, Mesolimbic, and Limbic Systems

A number of additional interconnected neural pathways have been implicated in maternal behaviour. Specifically, the connections of the olfactory system and the mesolimbic dopamine system with the limbic system have been found to be central to the onset and maintenance of maternal behaviour (Corter and Fleming, 2002; Numan and Stolzenberg, 2009). These pathways are responsible for stimulation, emotional behaviours, memory, and reward. Olfactory input into the limbic system inhibits maternal behaviour in nulliparous rats that have been continually exposed to foster pups, whereas disruption of olfaction significantly decreases latency to responsiveness from eight days to one (Mayer and Rosenblatt, 1975). Olfactory input acts on the amygdala of the limbic system, a brain region associated with fear and avoidance, indicating olfactory stimuli of the pups leads to avoidance and, potentially, fear in the nulliparous rat (Numan and Insel, 2003). This system provides the main mechanism for external pup related stimuli to influence the brain and support the plasticity needed for the development of maternal behaviour.

In contrast, the mesolimbic dopamine (DA) system is associated with reward, and is dependent upon the neurotransmitter dopamine (Numan and Stolenzberg, 2009; Stolzenberg and Numan, 2011). Significant structures within the mesolimbic DA system are the hippocampus, the VTA, the amygdala, and the nucleus accumbens (NA) (Li and Fleming, 2003). Disruption of maternal behaviour occurs when the VTA and the NA shell, but not the NA core, are lesioned (Li and Fleming, 2003; Numan and Stolenzberg, 2009). Further investigations outline the importance of

both dopamine 1 (D1) and dopamine 2 (D2) receptor subtypes within the NA shell (Parada, King, Li, and Fleming, 2008). Most researchers have only been able to isolate a disruption of maternal behaviour with D1, but not D2 receptors within the NA (Numan et al., 2005; Numan, 2006; Numan and Stolzenberg, 2009). Both D1 and D2 receptor antagonists have been shown to affect maternal behaviour within the MPOA. Specifically, D1 receptor antagonists inhibited maternal behaviours, whereas D2 receptor antagonists facilitated nursing behaviours (Miller and Lonstein, 2005). D1 and D2 receptor antagonists placed in osmotic pumps both disrupted maternal behaviours in a similar fashion; however, utilizing this method of infusion, D2 receptor antagonists inhibited maternal responsiveness for a period of up to seven days (Byrnes, Rigerio, and Bridges, 2002). While it appears certain that both receptors are involved in specific maternal behaviours, this area of research remains unclear as to the degree and specificity of their involvement. For future analysis, D2 receptors from the MPOA in the current study were targeted with antibodies for horseradish-peroxidase (HRP) staining immediately after tissue removal.

4.3 Synaptic Plasticity

It is evident from the literature that the onset of maternal behaviour is a significant event that is facilitated by both hormonal and neural input. Maternal behaviours, once elicited, are maintained for weeks and the experience affects future onset, regardless of hormonal influence (Fleming and Sarker, 1990). Clearly, lasting neural plastic changes occur following the initial development of maternal behaviour in rats. Changes in the brain after significant learning events and long-term potentiation are evident at the synaptic level (Weeks, Ivanco, Leboutillier, Racine, and Petit, 1999; Weeks, Ivanco, Leboutillier, Racine, and Petit, 2000). Evidence of plasticity has been found within the limbic structures of dams, such as the MPOA, and the amygdala (Fleming

and Korsmit, 1996). Investigation of neural plasticity within key regions of maternal circuitry can elucidate the relevance of specific structures involved in the onset and maintenance of maternal behaviours. Although synaptic changes were not examined in the current study, the relevant neural tissue was retained and prepared for future analysis.

5 Rationale

As discussed, hormones are significant modulators of maternal behaviour. The research project that forms the basis of this thesis investigated the effect of two key hormones, estrogen and progesterone, on the initial onset of maternal behaviour. Unlike previous studies, this current research focuses on maternal behaviours within the initial 24 hours post-parturition. The majority of studies investigate differences in behaviours after animals have displayed full maternal behaviours or a number of criteria, such as retrieval of all pups within an imposed time limit. These criteria prohibit the examination of specific behaviours except through latency or after the learning event has already been retained. There is clear evidence that estrogen plays a substantial role in the establishment of maternal behavior and this is especially true when it is examined in combination with other hormones. Progesterone was also chosen based on the findings that it significantly increases the efficiency of estrogen.

Previous studies investigating the interplay between steroidal and neural influences on maternal behaviours have typically used extirpation and replacement strategies that involve the surgical removal or chemical inhibition of endogenous activity within the research subject (Fleming and Corter, 2002). These investigations were fundamental in providing evidence for the role of steroidal hormones and neural regions in pregnancy, parturition, and maternal responsiveness (Terkel and Rosenblatt, 1972). Utilizing similar strategies, subsequent research has also been

conducted that induced maternal behaviour in virgin animals (Fleming, Cheung, Myhal, and Kessler, 1989; Doerr, Siegel, and Rosenblatt, 1981). Importantly, little research has been conducted with endogenous systems intact. In order to compare virgin animals to a naturally pregnant condition, all treatment groups in this thesis had intact endogenous systems. Previous studies comparing intact cycling virgins and ovariectomized virgins showed no difference in onset or expression of maternal behaviour in either group (Rosenblatt, 1967; Rosenblatt, Hinde, Beer, Busnel, 1988). These studies indicate that the induction of maternal behaviour in virgin animals is not dependent or mediated by naturally cycling hormones. Keyser-Marcus, et. al (2001) compared the structural changes of MPOA neurons of ovariectomized (OVX) animals without progesterone and estrogen (P+E) treatment, OVX animals with P+E treatment, late pregnant animals, late lactating animals, and diestrus-cycling virgins. OVX animals without P+E treatment, diestrus-cycling virgins, and late lactating animals all had similar and significantly smaller cell bodies than the late pregnant and OVX with P+E treatment animals. Taking the behavioural and neuroanatomical results of these studies into account, differences within behaviour and the MPOA of the current thesis project may be anticipated to be attributable to the treatment group and not to the endogenous cycling of the animals. Any behavioural or anatomical deviations, however, may be cross-referenced to the measured hormone levels in the blood. While this approach adds complexity due to the presence of naturally occurring hormone levels, this study was designed to measure and account for these changes and has the inherent comparative advantages that come from examining fully intact animals.

This study evaluated observed maternal behaviours in order to examine the extent to which hormonal manipulation induces responsiveness to pups. Comparisons were made between

hormonally intact pregnant dams, control nulliparous animals, and hormonally enhanced nulliparous animals. Animals were allocated into three treatment groups: pregnant dams, enhanced virgins, and control virgins. Half of the animals of each group were randomly placed in the no-pup condition to be utilized for future tissue analysis comparisons. This study tested the following hypotheses: 1) That more and potentially different maternal behaviours occur in enhanced virgin animals when compared to control virgins; 2) That maternal behaviours expressed by pregnant dams occur at higher incidences for all behaviours compared to enhanced and control virgins; and 3) That concentrations of progesterone in blood serum of the enhanced virgins are similar to pregnant dams and significantly higher than that of control virgins. Maternal behaviours examined included approach and care behaviours such as sniffing, grooming, hovering, and retrieval. Contrasting behaviours such as avoidance were also observed. During the hormonal enhancement phase of the study, progesterone levels were measured to ensure the efficacy of the procedure. Animals were sacrificed within twenty-four hours of initial onset to maternal behaviour, and neural tissue from the MPOA was removed for future analysis of ultrastructural plasticity. The MPOA was chosen due to its significant role within the neural circuitry, its high density of estrogen receptors, and its proximity to neuroendocrine input.

Methodology

1 Animals

Fifty-six female albino Wistar rats (twenty-four primiparous, gestational day [GD] 8; thirty-two nulliparous; all aged 57-64 days) were shipped from Charles River Labs, Quebec via airplane (see Appendix B for Animal Use Protocol Certificate; see Figure 1 for study design). Eight

animals were timed pregnant to serve as surrogate dams. Upon arrival, each animal was allotted a random code indicating treatment condition. Animals were housed in Ancare R20 Series polycarbonate cages with corncob bedding, enrichment huts, willow chew sticks, and high-calorie gel food. Normal rat chow and water was provided *ad libitum*. Animals received banana chips daily during normal checks and all procedures. Pregnant animals were placed in a separate holding room, adjacent to the virgin animals; holding rooms were placed on a reverse light cycle (12h:12h light:dark). Animals were given 5 days to habituate to their new environments (see Figure 2).

Pregnant	Enhanced Virgins	Control Virgins	Pregnant
With Pups	8 (6)	8 (6)	8 (6)
Without Pups	8 (7)	8 (7)	8 (7)

Figure 1. Study design. Behavioural analysis conducted on enhanced virgins, control virgins and pregnant rats exposed to pups (With Pups) and not exposed to pups (Without Pups); utilized for tissue analysis outlined in appendices. Target sample sizes are preceded by actual sample sizes.

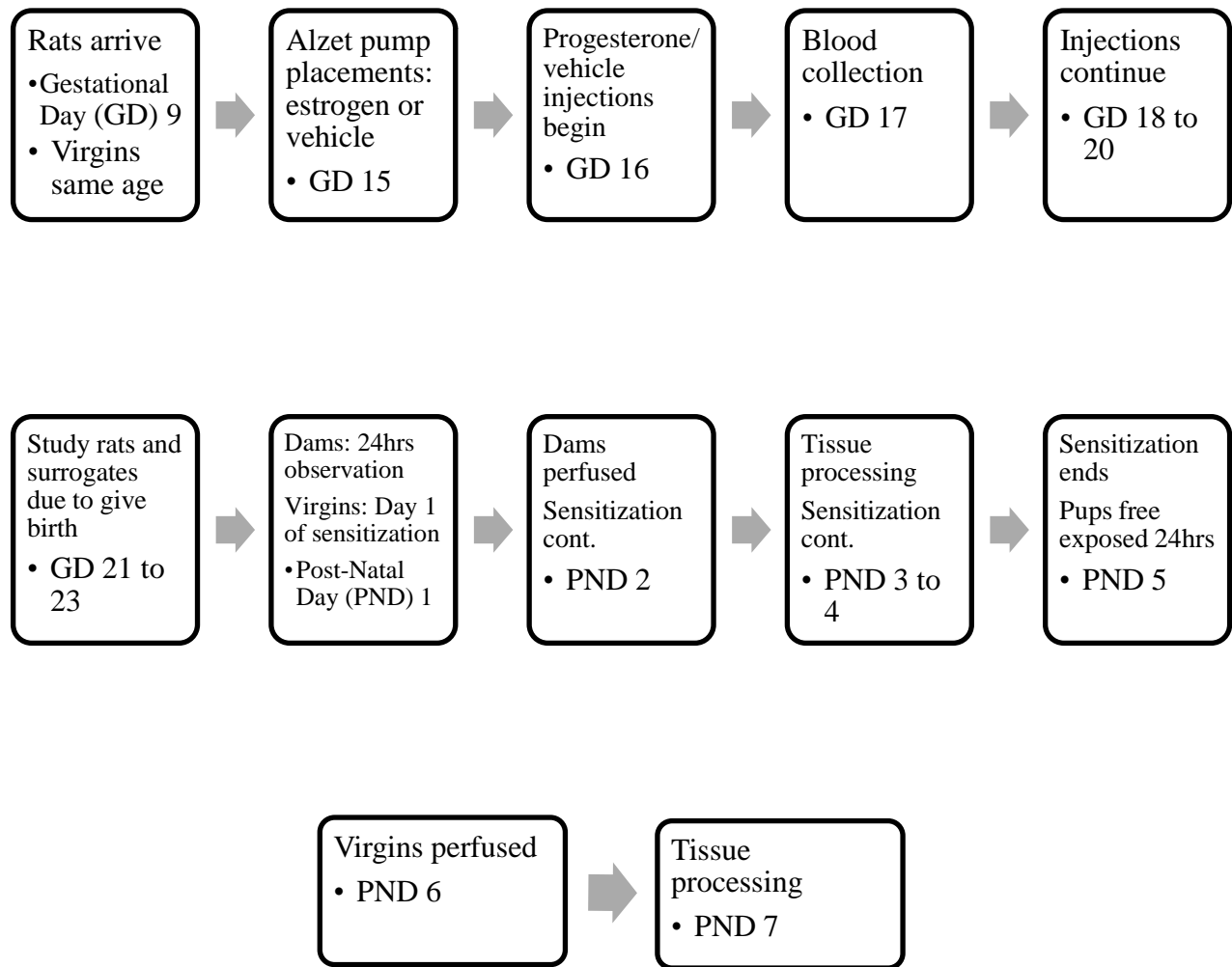


Figure 2. Project Timeline

2 Surgeries, Injections, and Blood Collection

On GD 15 experimental animals were given an injection of analgesic Meloxicam (1mg/kg) and were anesthetized in an induction chamber with isoflurane and oxygen (2-4%). A 1- inch square patch of fur was shaved between the shoulder blades and 2.5ml of sterile normal saline was injected subcutaneously. A 1-2cm incision was made between the shoulder blades. Using a

haemostat, a subcutaneous pocket was created under the skin, through the incision site, and an Alzet osmotic pump (flow rate of 0.11 µl/hr) filled with either Estradiol Benzoate (EB, 36 µg/100 µl vehicle) or peanut oil (vehicle) was placed under the skin (Mark, Augustus, Lewis, Hewitt, and Waddell, 2009; Mark, Smith, and Waddell, 2006). Pumps were primed with sterile saline prior to surgery. One or two surgical clips were used as needed to close the incisions, and Polysporin® was spread over the wound.

From GD 16, each animal received a subcutaneous injection of progesterone or vehicle daily, decreasing in dosage until parturition (10mg, 7.5mg, 5.0mg, 2.5mg [and 2.5mg of EB on the last day of injections]) to mimic the natural endogenous changes (Mark et al., 2009).

On GD 17, each animal was anesthetized with isoflurane and oxygen and the right hind leg was shaved. The saphenous vein was visualized and utilizing a lancet, punctured. Approximately 250 µl of blood was collected into an amber BD Microtainer. Blood samples were stored at 4°C overnight, centrifuged, and then frozen.

3 Sensitization

In order to minimize aggression of virgins towards foster pups, the following sensitization procedure was used. Once surrogate dams gave birth to full litters, pups were cross-fostered with other litters and randomly returned in litters of sixteen pups with each surrogate. Extra pups were euthanized via lethal inhalant exposure (isoflurane) according to animal care guidelines. Once the new litters were fed (visual check of milk band on pups' stomachs for confirmation: see Figure 3), eight pups from each surrogate were removed.

Large cotton Kimwipes® were placed at the bottom of 32 ounce Ziploc® Twist 'n Loc® containers which had approximately fifteen air holes drilled into the bottoms and lids. A handful of each individual virgins' bedding was placed on the Kimwipe® with four scented pups (pups were gently rolled in the respective virgin's bedding). A second Kimwipe® was placed on top of the pups and the lids were sealed (see Figure 4). Enrichment huts were removed from the virgins in the with-pup condition and the pup-containers were placed in the front of the cage, away from the virgins' nesting sites (see Figure 5). Freshly fed and scented pups were replaced every 4-6 hours for a period of 5 days.



Figure 3. Visible milk band on pup



Figure 4. Pups placed in cup, surrounded by soft tissue.



Figure 5. Virgin rat with pups in container

On day 5, rats were placed in a clean cage and two scented pups were free-exposed; one per corner in the front of the cage. The experimenter remained close to the cage for a period of 15 minutes to allow for quick removal of pups upon signs of aggression. If aggression was observed, pups were removed immediately and euthanized if injured. Aggressive animals were excluded from observations, based on failure to exhibit onset to maternal behaviour by day 5 (actual sample sizes, see figure 1).

4 Observations

A series of infrared cameras, one per experimental animal, were placed in the holding rooms to record 10 minutes of activity every 2 hours (see Figure 6). Upon parturition, dam litters were removed, cross-fostered with other litters, and four random pups were returned to each animal in the with-pup condition. A total of twelve recordings were made from the time pups were returned (or from the moment of free-exposure in the virgin conditions) during a period of 24 hours.



Figure 6. Infrared camera setup

A condition-blind experimenter reviewed the videos and recorded behaviours. Avoidance of pups, nursing, and hovering behaviours were measured using a stopwatch to determine the total minutes spent performing these behaviours (see Figure 7). Pup retrieval, grooming, sniffing, and nest building behaviours were tallied by incidence. Due to aggression expressed by some virgin animals in both enhanced and control conditions, some animals had their foster pups removed and were unable to be included for behavioural observations. After the 24-hour observation period, animals were given a lethal injection of sodium pentobarbitol (100mg/kg) and were perfused intracardially (see appendix B).



Figure 7. Dam nursing her litter

5 Progesterone Extraction

Thirty-two out of forty-four blood samples contained enough plasma to be usable (9 in the pregnant condition, 12 enhanced, and 11 control). Fifty μl of plasma from each sample was combined with petroleum ether and then vortexed (Neogen, 2014). The organic phases were transferred into glass tubes and evaporated using nitrogen gas (see figure 8). Residues were extracted with 250 μl of extraction buffer, vortexed, and assayed into 50 μl duplicates. Samples were measured at 450nm (1N HCl) using a Perkin Elmer Victor1 microplate reader.

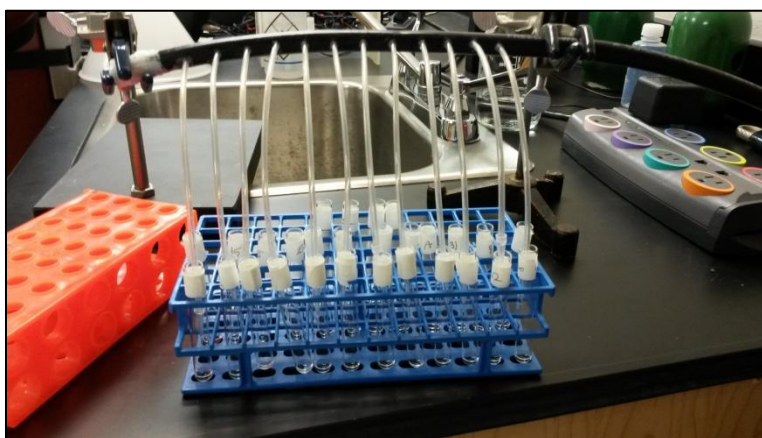


Figure 8. Nitrogen gas streaming

6 Statistical Analyses

Statistical analyses using IBM SPSS 19 were conducted to evaluate behavioural differences between and within groups. Repeated measures analysis of variance (ANOVA) was conducted for the observed behaviours including hovering, grooming, sniffing, retrieval, nest building, and avoidance. A one-way ANOVA was conducted to confirm that progesterone concentration levels were accurately matched for enhanced virgins to pregnant controls for animals included in

the behavioural analysis. A linear regression analysis was performed to calculate the coefficient of determination. P-values were set at 0.05 for significant findings.

Results

1 Progesterone concentrations

Progesterone delivery was confirmed using ELISA analysis. Concentrations of progesterone in blood samples were interpolated based on the approximate concentrations provided by the ELISA analysis. Concentrations of progesterone between the groups were not significantly different [$F(2,15) = 0.590$, $p = 0.560$; see figure 9]. The difference between the enhanced group ($M = 49.01\text{ng/mL}$, $SD = 15.81$) and the pregnant group ($M = 45.00\text{ng/mL}$, $SD = 20.01$) was not found to be significant, $p = 0.626$. Progesterone concentrations between the enhanced and control virgin groups ($M = 38.89\text{ng/mL}$, $SD = 15.14$) was also not found to be significant, $p = 0.290$, and likewise between the control virgin and pregnant groups, $p = 0.558$.

Due to small sample sizes and non-significant results, Cohen's d was performed to evaluate the effect size between enhanced virgin and pregnant groups to ensure the trend in the data confirmed progesterone serum levels ($d = 0.223$, $r = 0.111$). The magnitude of the effect was small, indicating low practical significance. Cohen's d between enhanced and control virgin groups was moderate ($d = 0.654$, $r = 0.311$) and between control virgin and pregnant groups ($d = 0.344$, $r = 0.17$), indicating high to medium practical significance between these groups.

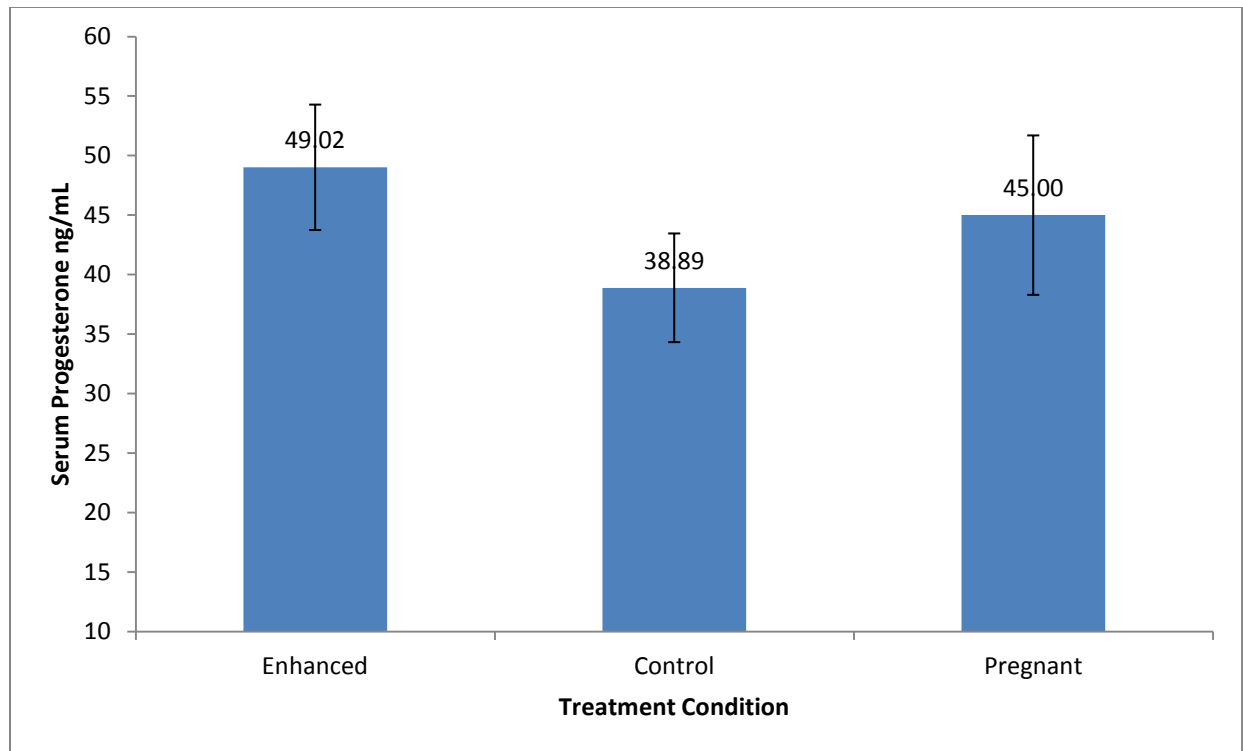


Figure 9. Progesterone Serum Levels.

2 Behavioural Analysis

Repeated measure ANOVA [Split plot factorial 3.12 (Between-subjects, Group: 1 - control virgins, 2 - enhanced virgins, 3 - pregnant; Within-subjects, Behaviour: 1 - first observation, through 12 - twelfth observation)] analyses were conducted to evaluate specific maternal behaviours over the course of twenty-four hours. There was a significant interaction with grooming behaviour and group conditions: $\lambda = 0.008$, $F(22,10) = 4.476$, $p = 0.009$, $\eta^2 = 0.908$. Significant differences in grooming behaviour were observed between hours one and nine inclusive for pregnant dams compared to both control (mean difference = 1.431, $p = 0.001$) and enhanced virgin groups (mean difference = 1.389, $p = 0.001$; see figure 10).

Hovering and crouching behaviours observed had a significant group effect: $F(2, 15) = 706.569$, $p = 0.001$, $\eta^2 = 0.989$. Pregnant dams differed from control virgins (mean difference = 8.986 minutes, $p = 0.001$) and from enhanced virgins (mean difference = 8.542 minutes, $p = 0.001$) for all observations (see figure 11).

Pup-retrieval interaction with group conditions was not significant: $\lambda = 0.571$, $F(8,24) = 0.969$, $p = 0.483$, $\eta^2 = 0.244$, as was the between subjects effects: $F(2,15) = 0.953$, $p = 0.408$, $\eta^2 = 0.113$. Nest building interaction with group conditions was also not significant at the: $\lambda = 0.161$, $F(18,14) = 1.160$, $p = 0.394$, $\eta^2 = 0.599$. Further, pup-sniffing interaction with group conditions was also not significant: $\lambda = 0.135$, $F(22,10) = 0.781$, $p = 0.70$, $\eta^2 = 0.632$.

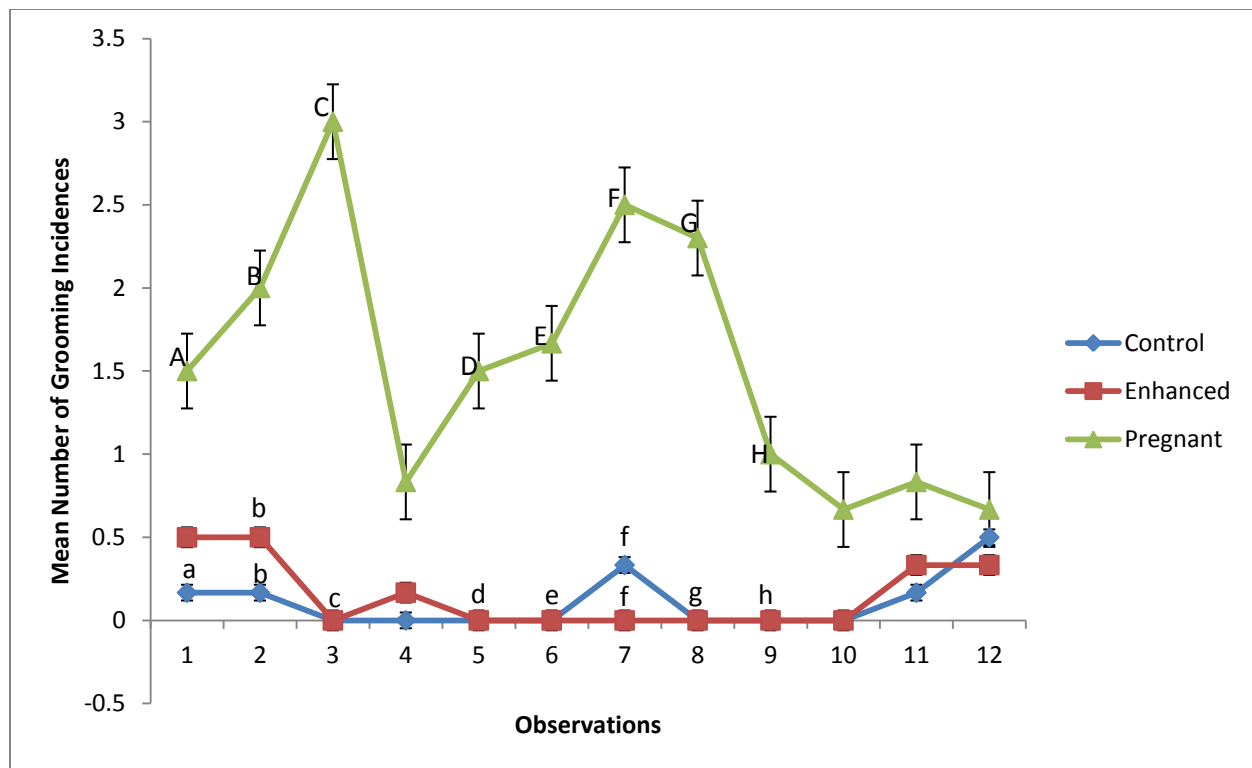


Figure 10. Grooming behaviour observed for 24hrs post-parturition. For observations 1 through 9 significant differences noted between Pregnant and Control (MD 1.431, $p = 0.001$) and between Pregnant and Enhanced (MD 1.389, $p = 0.001$) and are indicated by large and small letters.

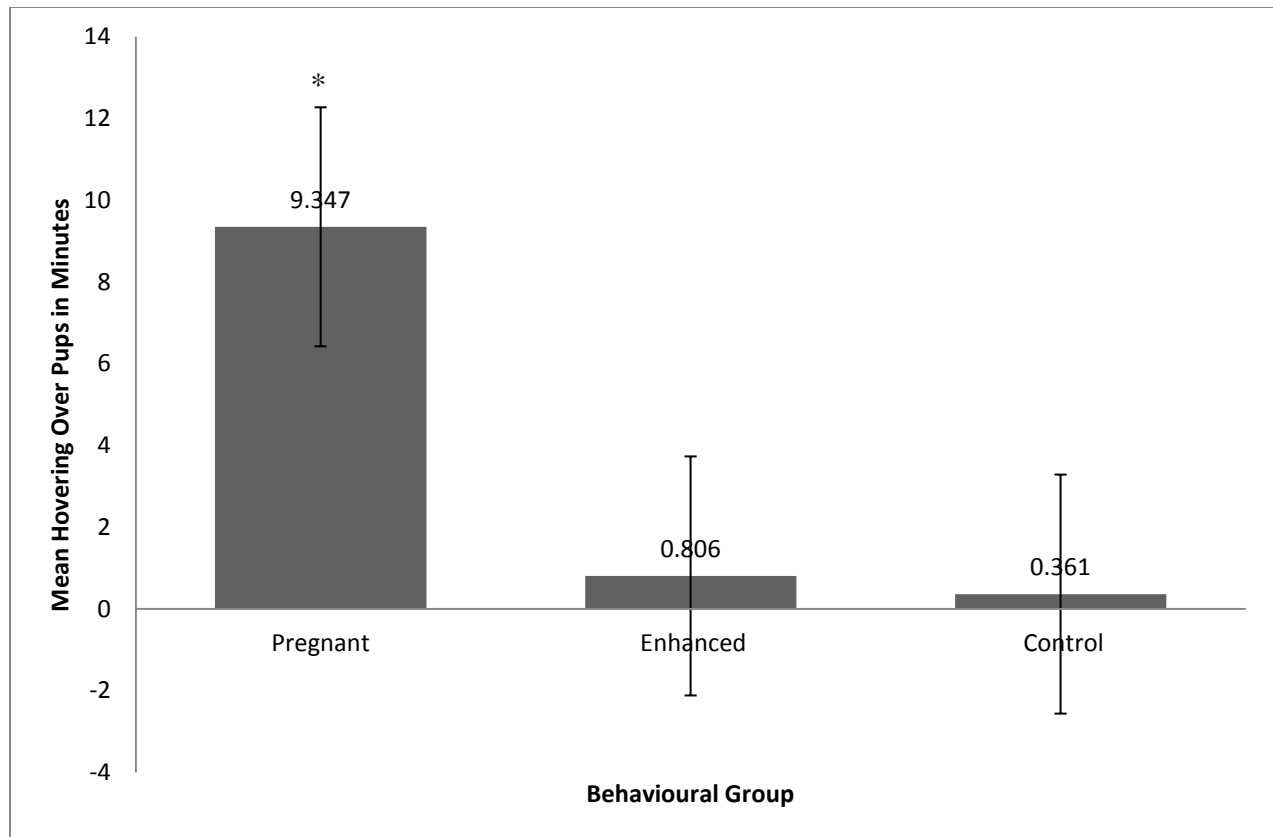


Figure 11. Hovering behaviour observed for 24hrs post-parturition (Pregnant vs Enhanced MD = 8.542 min, $p = 0.001$; Pregnant vs Control MD = 8.986 min, $p = 0.001$)

There was a significant main effect between avoidance behaviour and group conditions: $F(2,15) = 178.260$, $p = 0.001$, $\eta^2 = 0.960$. Both enhanced virgins (mean difference 8.313 minutes, $p = 0.001$) and control virgins (mean difference 8.646 minutes, $p = 0.001$) avoided pups significantly more than pregnant dams for all observation periods (see figure 12).

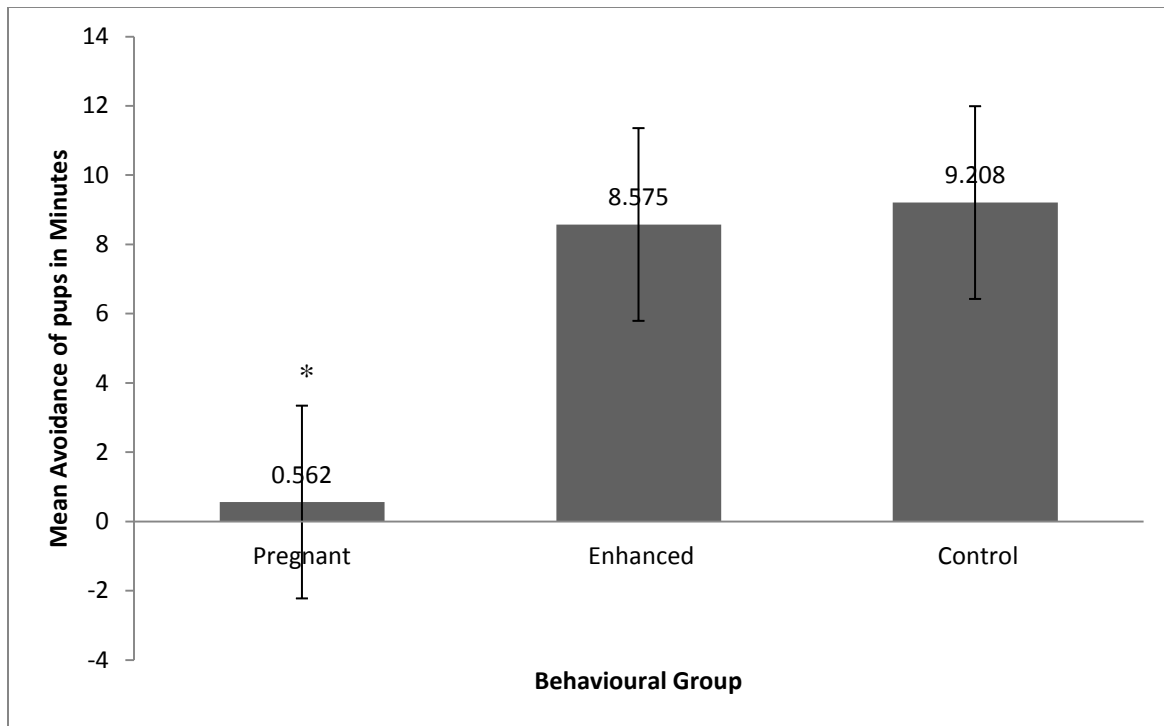


Figure 12. Avoidance behaviour observed for 24hrs post-parturition (Pregnant vs Enhanced: MD = 8.313min, $p = 0.001$; Pregnant vs Control MD = 8.646min, $p = 0.001$)

In order to examine the level of maternal behaviour that may be accounted for by progesterone, a scatterplot was created comparing the maternal behaviour and progesterone serum levels for the three group conditions (see figure 13). The linear regression values indicates 36% of the maternal behaviours observed in the enhanced condition can be accounted for by progesterone, 30% for the pregnant condition, and 4% for the control.

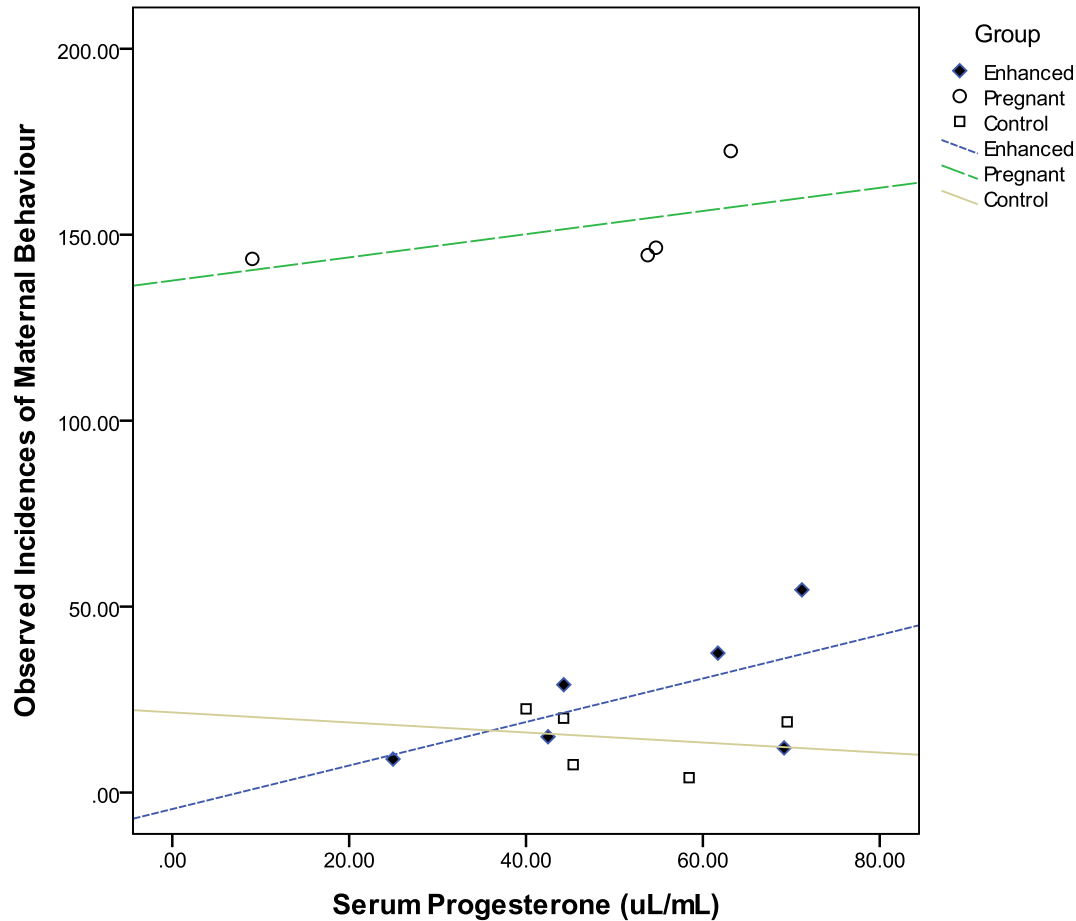


Figure 13. Association between observed maternal behaviours and serum progesterone levels. R^2 Enhanced = 0.36; R^2 Pregnant = 0.30; R^2 Control = 0.04

Discussion

1 Summary of Results

This study examined the effects of steroidal hormones on initial maternal behaviour by manipulating estrogen and progesterone levels in hormonally enhanced intact virgins and compared them with normal dams and control virgins. The research design tested the primary hypothesis that hormonally enhanced virgins, with intact endogenous systems, would be initially more responsive than matched controls. Behavioural results fail to support this hypothesis with

no significant differences observed between drug and vehicle conditions in the virgin female rats. As predicted, there was a significant difference between pregnant dams and both of the other conditions, but not for all maternal behaviours. A number of behaviour differences did not reach significance across groups, such as sniffing, nest building, and retrieval. The maternal behaviours where differences were observed included hovering or crouching, avoidance, and grooming. Previous research has shown that virgin rats have lower nest building scores and longer latencies to retrieval than dams (Fleming and Rosenblatt, 1974). Both hovering and grooming behaviours are activities that become stronger with longer pup exposure once tolerance or onset begins. Hovering and nursing behaviour have previously been noted to be a responsive activity, stimulated by nuzzling pups, that is mediated by a different neural circuit (Numen and Stolzenberg, 2009). These previous studies partially explain the current results because they indicate that retrieval and grooming behaviours are not strongly expressed until full onset of maternal behaviour.

The third hypothesis stated that regardless of endogenous hormonal fluctuations, progesterone levels in the drug condition would be similar to that of pregnant dams. Concentration levels were confirmed as a significant trend. The concentrations of progesterone in the enhanced virgins were similar to the endogenous levels of pregnant dams. Appropriately, the concentration of endogenous progesterone in the control virgins was lower than that of the enhanced virgins and pregnant dams. Measures of the effect sizes and relationship strengths were evaluated with Cohen's *d*. Drug and pregnant groups differed by 0.0937 standard deviations, a small effect, confirming the progesterone concentrations were similar and an appropriate dose was circulating in the enhanced virgins.

2 Limitations

The sensitivity of the results may be weak with group sizes of $n = 6$ and $n = 7$. A number of animals were removed from the study due to exclusionary criteria and necessary ethical restrictions. For instance, one animal within the pregnant condition failed to deliver any pups and, upon examination after euthanization, it was apparent that she had a constriction in her uterus. One surrogate animal began displaying signs of serious distress (erector pili, porphyria, odd posturing, lack of interest in food or water) and was euthanized to prevent suffering. Dissection of her abdomen revealed constriction in her uterine cavity and an infection. To ensure the availability of enough foster pups, a pregnant dam previously designated as a research animal was removed from the study to replace a lost surrogate dam.

Every effort was made to maintain environmental conditions to minimize stress on the animals since this can disrupt parturition and pup acceptance. Despite these efforts, an additional six rats (4 enhanced, 2 control) were excluded from behavioural testing because they attacked foster pups within twenty minutes of free introduction. Prevention of pup-attacks could have been mediated with a longer sensitization period; however, due to the necessity to capture the brain within twenty-four hours of the primary behavioural acquisition, animals were tested at the lower end (five days) of reported latencies to maternal onset in virgin rats (Cosnier and Couturier, 1966; Rosenblatt, 1967). The higher end (seven days) of sensitization would potentially have prevented pup aggression, saving both the pups and the behavioural data but another complication may have occurred. Specifically, if some animals within the groups were initially maternal at day five, their brains would no longer be comparable to animals that took the full seven days to become responsive. Further, probing animals at day five and retesting later could produce a confound of re-initiating primary contact, whereas other animals would only receive

one initial contact. Observations from pilots and previous experience within the laboratory suggest previously aggressive rats are unlikely to become maternal in subsequent probe trials (Weeks et al., 2009).

Loss of progesterone samples due to insufficient blood withdrawal (Animal Care Committee approved a three prick cut-off; see Appendix A) reduced the sensitivity of the analysis and effect sizes were examined to evaluate the strength of the concentration differences between the groups. To increase the efficacy of hormonal enhancement, previous studies reported exogenously enhancing animals for a period of up to thirteen days (Bridges, 1984). To ensure animals arrived pregnant and had sufficient time to habituate to their new surroundings, surgeries were held on gestational day fifteen, thereby reducing the hormonal priming to five days. Estrogen concentrations utilizing similar endogenous enhancement methods to this study have previously been confirmed to match pregnant controls (Mark, Smith, and Waddell, 2006). Estrogen concentrations are low, measurable only in picograms per millilitre, requiring a laboratory equipped for radioimmunoassays. Unfortunately, this technique was not available for the current study. Regardless of the inability to test endogenous and exogenous estrogen levels estrogen concentrations during oestrous cycles have been demonstrated to not significantly alter the onset to maternal behaviour in any phase (Rosenblatt, Hinde, Beer, and Busnel, 1980).

Another potential limitation of this study involved the type of osmotic pump utilized. The pumps chosen for this study had a forty-two day diffusion reservoir to eliminate the need to place animals back under anesthesia to remove or replace them. This lengthy diffusion potentially reduced the validity of testing the residual volumes of the pumps to ensure exact diffusion rates. Osmotic pumps absorb interstitial fluid in order to create a pressure and diffuse the solution inside the reservoir and unless completely discharged, the weight of the pumps would be

inaccurate. Pumps were extracted within six to twelve days of insertion, which was relatively early compared to the forty-two day maximum. As a result, post-perfusion weights of the pumps were higher than when the pumps were inserted. This indicates the pumps had an influx of interstitial fluid during the six to twelve day period they were in place. In order to confirm an accurate diffusion rate for this short implant duration, a test pump filled with vehicle and dye was placed in a simulated subcutaneous environment. The test revealed that despite the inability to use the weight differential as confirmation, the pumps did in fact discharge the correct quantity of estrogen solution into the rats.

3 Future Directions

Further investigation of the ultrastructural synaptic structures within the MPOA will be conducted with a transmission electron microscope to determine if the drug condition influences synaptic plasticity within this region. Although there were no observable significant differences in behaviour between hormonally enhanced virgins and controls, this does not necessarily mean that no neuronal or synaptic changes occur within specific brain regions. Synaptic analysis may identify changes within the MPOA that occur based on hormonal manipulation and precede an eventual behavioural change. Utilizing the methods described by Weeks et al. (1999), this future analysis will involve synaptic quantification, synaptic type (excitatory versus inhibitory synapses), and synaptic structure (perforations, length, curvature). A proportionate analysis of highlighted synapses (D2 receptors) will also be conducted for this region. This analysis may reveal subtle differences in neural connectivity and plasticity within the MPOA of the maternal circuit that are not apparent in the behavioural analysis. Furthermore, if the analysis reveals few or no significant differences in synaptic ultrastructure, a follow up experiment may be conducted with ovariectomized virgin groups to determine if this factor has impacted the results.

4 Conclusions

Estrogen and progesterone have been established in the literature to mediate latency to maternal behaviour, but have also been shown to not be a requirement for the onset of maternal behaviour (Doerr, Siegal, and Rosenblatt, 1981; Rosenblatt, 1967). Many studies include their own operational definition of onset to maternal behaviour. This typically involves full-onset when animals display a number of criteria-based behaviours or the retrieval of all pups to a nesting site (Pedersen and Prange, 1979). The present study examined the extent of maternal responsiveness twenty-four hours from the initial onset of maternal behaviour operationally defined as tolerance to pups and a lack of aggression. The current results indicate that early maternal behaviours, once the animals are tolerant of the foster pups, are not different between drug (estradiol benzoate and progesterone) or vehicle conditions in virgin animals and that the onset of these behaviours showed a slow progression. Pregnant dams, however, displayed immediate and varied behaviours as you would expect in the natural condition. While it is important to acknowledge the potential limitations of the study discussed above, these results support previous studies that hormones interact with other systems such as neural circuits to initiate maternal responsiveness, but not to enhance maternal behaviour. The results of this study provide a basis for future investigations of specific behavioural and, in conjunction with the results of the future tissue analysis, synaptic changes that occur at early stages of maternal learning.

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Appendices

Appendix A. Standard Operating Procedure for Saphenous Vein Puncture

Procedure Title: <u>Blood collection from the lateral saphenous vein in rats</u>	Procedure #: PR-08		
	Effective Date: June 2015	Revision Date:	Review Date: June 2018
	Pages: 5		
Purpose: Describes the methods for blood collection in rats using the lateral saphenous vein. This SOP follows the UBC and CCAC guidelines for the collection of blood in laboratory rodents.			
Policy: to follow CCAC and Nipissing University guidelines			
Authorized User(s): Researchers, animal care technician, veterinarian and trained student researchers listed on animal care protocols			
Revised by:			

Materials

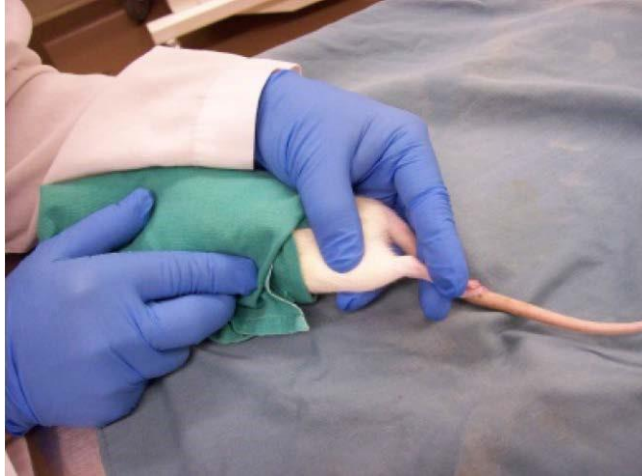
- Appropriate animal restrainer (clean cloth)
- Clippers or depilatory cream (Nair®)
- 70% Isopropyl alcohol
- Cotton swabs
- Glycerin or petroleum jelly (Lacrilube works)
- Blood collection tube
- 25-26 gauge needle or animal lancet appropriately sized for the animal
- 2 x 2" gauze
- Anesthesia, if required

NOTE:

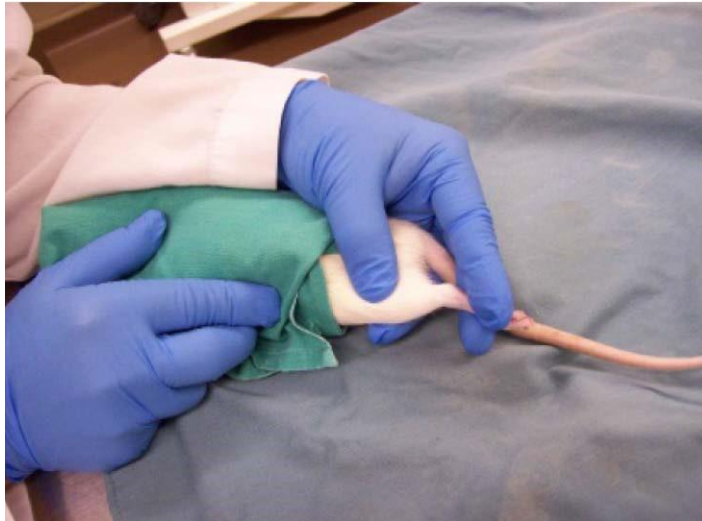
This procedure makes it very difficult to restrain the rat and it is quite stressful for them. Anesthesia is recommended (SOP A-01) unless there is a high level of proficiency with this technique.

Procedure

1. Choose an appropriate restrainer (typically a clean cloth/towel) or anesthetize the rat.
2. If using a restrainer, the animal is held in the restrainer head first so that only the rear legs and tail are free. The rear leg can be stretched out into a natural position.



3. To secure the animal and elevate the vein, the skin on the upper thigh is gently but firmly squeezed, using the same hand that is holding the restrainer.

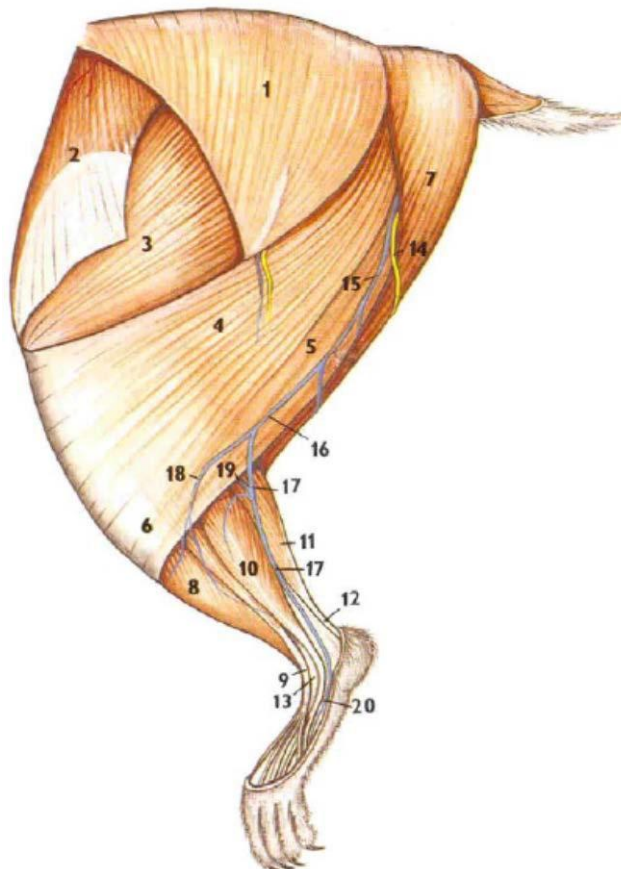


4. The hair is removed either by clippers or by using a depilatory cream and swabbing with 70% alcohol. Depilatory cream should not be left in contact with the skin for more than 1 minute and can be wiped away with alcohol.

5. Swab the skin with a small amount of alcohol to help visualize the vein.



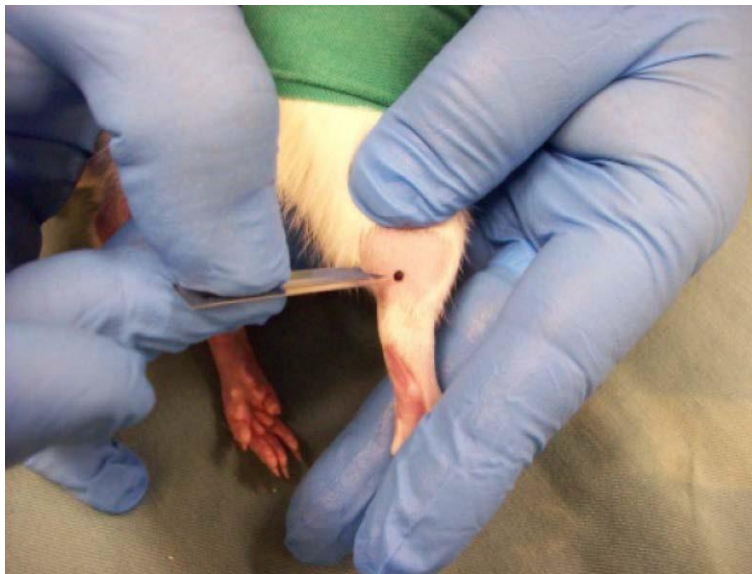
6. Locate the lateral saphenous vein (see number 17 below).



7. A thin film of a bland ointment such as Vaseline® or glycerin can be applied to prevent blood from seeping into the fur and allow for blood drop formation.



8. Using a 25-26 gauge needle or an animal lancet, puncture the vessel at a 90° angle at the most proximal (closest to the body) visible site.



9. Collect the sample into your collection tube ensuring you do not exceed the allotted 10% blood volume loss.



10. Use a dry piece of gauze to apply pressure to the puncture site while releasing pressure on the upper thigh until bleeding stops.
11. Remove the rat from the restrainer and place it back in its cage.
12. Monitor the animal for 5-10 minutes to ensure hemostasis (bleeding has stopped).
13. For repeat samples, the scab may be brushed off with a dry piece of gauze or a new puncture site can be made distal to the previous site (towards the foot).

Calculations

As a general rule most mammals have close to 70ml per kg of circulating blood volume. 10% of this volume (7 ml/kg) can be taken from healthy animals without deleterious effects. This amount can usually be removed every 2-3 weeks.

A volume of 250 μ L can readily be collected using the lateral saphenous technique in adult rats.

Single or serial blood samples can be collected from rodents without anesthesia using the lateral saphenous vein.

References

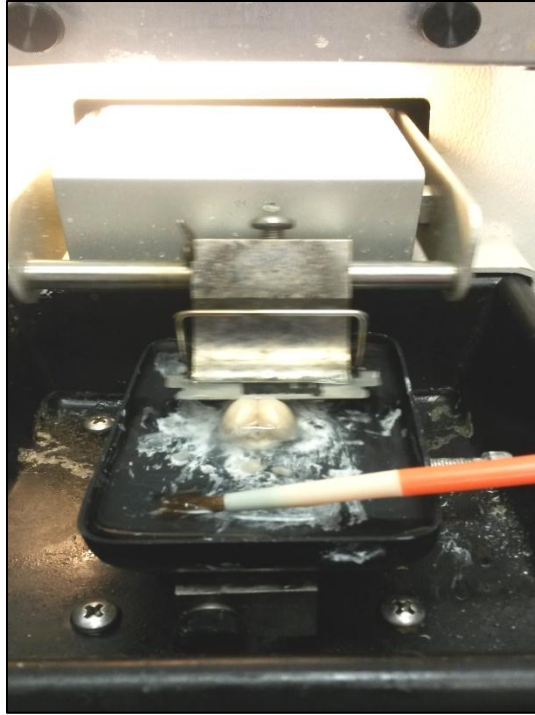
1. Canadian Council on Animal Care (CCAC) guidelines (www.ccac.ca)
2. UBC Animal Care Committee; Policy on Acceptable Methods of Rodent Blood Withdrawal: ANIMAL CARE POLICIES, SOPs & GUIDELINES | Office of Research Services. "Blood Collection from the Lateral Saphenous Vein in Mice and Rats SOP"

Appendix B. Tissue Preparation

1 Perfusions, Brain Dissection, and Sectioning

After the 24-hour observation period, animals were given a lethal injection of sodium pentobarbitol (100mg/kg). Intracardial perfusions were performed utilizing a 50ml heparin prewash solution and 500ml of 2% paraformaldehyde and 3.75% acrolein. Each perfusion lasted for a total of 20 to 30 minutes. Animals were then placed in biohazard bags and stored at 4°C for 2 hours. Whole brains were dissected by hand and placed in 0.1M phosphate buffer (PB).

Using a Pelco® Vibratome, brains were sectioned into 500µm thick slabs (see Figures below). The MPOA was visualized by referencing *The Rat Brain* atlas to confirm target structures and measurements (Paxinos and Watson, 2006). Three blocks of MPOA tissue were removed from each hemisphere, placed in a small glass vial with 0.1M PB, and labelled according to animal code, area, and hemisphere; for example 18mpoaR.



Brain sectioning with a vibratome



Brain sections from vibratome

2 Histochemical Processing

The subsequent histochemical process was adopted from a procedural text (D. Lane, personal communication, November 24, 2014; Milner, Waters, Robinson, & Pierce, 2011). In order to protect the integrity of cellular membranes, all histochemical processing took place immediately after perfusions. Subsequent tissue analysis will be conducted in the future.

Tissue blocks were placed in sodium borohydride for 30 minutes to remove unbound aldehydes and were then rinsed with PB until the tissue stopped reacting (bubbles disappear, tissues sink to bottom of vials). The tissues were then rinsed in 0.1M Tris saline (TS) and incubated for 30 minutes in TS with 0.5% bovine serum albumin (BSA) to block nonspecific staining. The BSA prevents cross reactivity between primary and secondary antibodies (Milner et al., 2011).

Tissues were rinsed in 0.1M TS and then placed in the primary antibody (dopamine 2, Abcam® DRD2) and incubated on a shaker for 24 hours at room temperature and then another 24 hours on ice.

Tissues were rinsed in 0.1M TS and were placed in the secondary antibody (biotinylated anti-rabbit) for 30 minutes. An Elite Vectastain® ABC (avidin-biotin-complex) kit was then used for peroxidase labelling. The tissues were incubated in the ABC solution for 30 minutes and were then washed in 0.1M TS to remove excess solution. Peroxidase labelling procedures were then finalized with a diaminobenzidine reaction lasting for approximately 6 minutes. Tissues were finally rinsed with 0.1PB.

To prepare tissues for electron microscopy, they were placed in 2% osmium tetroxide for 1.5 hours. Increasing concentrations of ethanol baths were then conducted to dehydrate the tissue over a period of a 2.5 hours, followed by 4 days of Spurr's Resin infiltration (Spurr, 1969).

Tissue blocks were then aligned within plastic capsules along with their respective codes and baked overnight at 60°C.

Appendix C. Animal Use Protocol Certificate



Laurentian University
Université Laurentienne

Protocol Certificate/ Certificat de Protocole

Certificate number/ Numéro de certificat : 2015-03-01

Name/ Nom: Céline Boudreau-Larivière

Title / Titre : Maternal neuroendocrinological manipulations and synaptic plasticity in the rat

Animals used / Animaux utilisés : rats

Approval date / Date d'approbation : April 8, 2015

Expiry date / Date d'échéance : April 8, 2016

A handwritten signature in black ink, reading "Dave MacLean".

Researcher's copy / Copie du chercheur
